

# Castration-induced apoptosis in the rat ventral prostate is associated with increased expression of genes encoding insulin-like growth factor binding proteins 2,3,4 and 5

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## Abstract

Insulin-like growth factor binding proteins (IGFBPs) have recently been demonstrated to act as regulators of apoptosis *in vitro* in both prostate and breast cancer cell lines. We show here that gene expression of IGFBP-2,-3,-4 and -5 increase rapidly in the rat ventral prostate following castration. Increases in IGFBP mRNA levels were detectable by Northern blotting by 6 hours and reached 5 to 10 fold of control levels at 72 hours after castration. Apoptosis in the ventral prostate, as detected *in situ* by the TUNEL method, was also induced as early as 6 hours after castration. TRPM-2/clusterin, a gene known to be associated with involution of the prostate, was not detected in sham castrated controls but was expressed by 24 hours following androgen ablation. IGF-I mRNA levels increased to 160% of control values within 6 hours following castration, then decreased gradually over the next 72 hours to 35% of control. Affinity labelling experiments demonstrated that IGF-I receptor levels increased initially after castration with peak binding at 24 hours, then declined to levels lower than control. These results suggest that rapid induction of IGFBPs in the rat ventral prostate following androgen ablation may play a role in apoptosis and involution of the prostate gland.

Androgens are required for normal prostate growth and function (1) and androgenic influences are involved in both benign prostate hyperplasia and prostate cancer (2,3). Although androgen deprivation is commonly used in the management of prostate cancer and is currently being evaluated as a strategy for prostate cancer chemoprevention (4), the molecular mechanisms underlying androgen effects are incompletely described.

Castration-induced androgen deprivation triggers a sequence of events which activates apoptotic cell death of the androgen-dependent epithelial cells within the rat ventral prostate ultimately resulting in the involution of the gland (5). Regression of the prostate begins within a day after castration and corresponds with a rapid decrease in serum testosterone, and more importantly, a gradual depletion of 5 $\alpha$ -dihydrotestosterone in prostate tissue (5). This system is therefore suitable to study molecular mechanisms responsible for apoptosis induced by androgen-deprivation.

Insulin-like growth factor I (IGF-I), which has been shown to protect cells from apoptosis (6), plays an essential role in normal prostate physiology (7). IGF-I is mitogenic for human prostate epithelial cells *in vitro* and its action is mediated through binding to the IGF-I receptor (8). Observations that IGF binding protein-3 (IGFBP-3) inhibits IGF-induced proliferation of normal prostate epithelial cells and that serum levels of certain IGFBPs are changed in patients with prostate cancer (9,10), suggest that IGFBPs (reviewed in (11)) are important modulators of IGF activity in the human prostate. More recent observations (12,13) suggest that IGFBPs may also play direct roles in regulation of proliferation and apoptosis.

While IGFs have been recognized as antiapoptotic factors for some time, it has only recently been demonstrated that IGFBPs can regulate apoptosis *in vitro* (12,14). We undertook these experiments to study the gene expression of IGFBPs during involution of the rat ventral prostate after castration.

## Materials And Methods

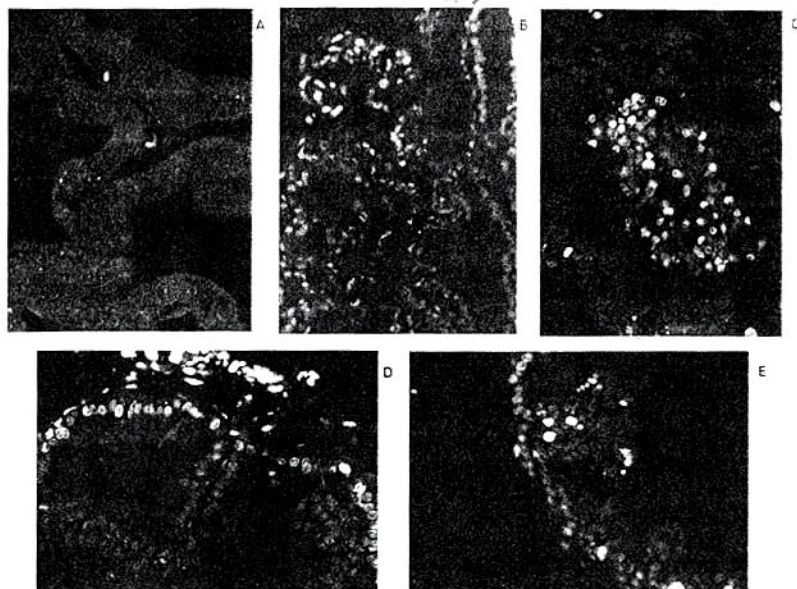
**Animals.** All animal studies were conducted in accordance with local humane animal care standards. Male Sprague-Dawley rats weighing 350-400g (Charles River, Quebec) were anaesthetized with pentobarbital (45 mg/kg body weight) and either castrated or "sham" castrated (scrotal incision only). Animals were sacrificed after 6 h and then every 24 h for 4 days. The ventral prostate was collected. Part of the tissue was fixed in 10% buffered formalin for paraffin embedding and the remainder was immediately frozen in liquid nitrogen for isolation of RNA and membrane fractions.

**Northern Blot Analysis.** Total RNA was isolated from ventral prostate using the RNeasy B method (Tel-Test). Total RNA (50 $\mu$ g) was fractionated on 1% agarose gel and transferred onto Zeta-Probe membrane (Bio-Rad) in 50 mM NaOH. The cDNAs for TRPM-2/clusterin (15), IGF-I (16) and IGFBP-2,3,4 and 5 (17) were labelled with [ $\alpha$ -<sup>32</sup>P]dCTP using T7 Quick-Prime kit (Pharmacia). Prehybridization, hybridization and washing were performed as previously described (18). Blots were subjected to autoradiography with intensifying screen at -80°C. Northern blotting was repeated on RNA from three sets of individual rats. Results were similar and all blots shown are from one representative filter which was stripped and rehybridized with the various probes. Quantitative analysis of gene expression was accomplished by averaging densitometric scanning of three autoradiograms per gene and results were corrected for minor loading differences by normalizing to 28S rRNA.

**IGF-I Receptor Binding Assay.** Cell membrane extracts were prepared from ventral prostate tissue as described (19). Membrane extracts containing 100 $\mu$ g of membrane protein were incubated with [<sup>125</sup>I]IGF-I (800,000 cpm) in PBS containing 1% BSA for 1 h at room temperature. Ligand was then crosslinked to receptors by incubation with 0.3mM disuccinimidyl suberate for 1h at 4°C followed by quenching with 0.5M Tris, pH 6.8. The membrane was pelleted by centrifuging for 45 min at 4°C. Proteins were separated by SDS-PAGE in reducing conditions followed by transfer to nitrocellulose membrane and autoradiography.

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**Figure 1.** Apoptosis in the rat ventral prostate following castration. The TUNEL method was used to detect apoptosis *in situ* in formalin-fixed, paraffin embedded sections of ventral prostate tissue. The sections were photographed using an FITC filter in a fluorescence microscope at a magnification of 320X or 200X (B). The nuclei of apoptotic cells contain fluorescein-labelled DNA and appear bright. Assays were performed on at least three independent tissue sections. Results from a representative assay including ventral prostate tissue from sham castrated rats (A) and 6 (B), 24 (C), 48 (D) and 72 (E) h after castration are shown.

**Quantitation of Apoptosis.** Apoptosis was detected in formalin-fixed, paraffin embedded tissue sections using the ApoAlert DNA fragmentation assay (Clontech) which is based on the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method. 5µm sections were prepared and treated according to the manufacturer's instructions. Apoptosis was visualised and photographed under a fluorescent microscope equipped with an FITC filter. Labelling indices were obtained by counting the number of labelled cells among at least 1000 epithelial cells per region and expressed as percentage values.

### Results

#### *Apoptosis in the ventral prostate induced by castration*

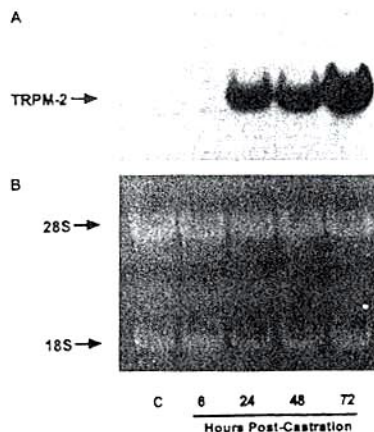
It has been known for some time that programmed cell death is activated in the rat ventral prostate after castration (5). TUNEL assays were performed on ventral prostate sections in order to make comparisons between the extent of apoptosis and changes in gene expression at the various time points. The lack of signal in prostate from sham castrated controls confirms minimal apoptosis in the gland prior to castration (Figure 1A). At 6 h following castration, ~65% of cells are apoptotic and fluoresce with an intensity related to their degree of DNA fragmentation (Figure 1B). By 24 h, ~80% of cells are apoptotic (Figure 1C) and the number of apoptotic cells in the ventral prostate reaches ~95% by 72 h following castration (Figure 1D,E).

Northern blot analysis was used to confirm the expression of TRPM-2/clusterin, a gene known to be associated with involution of the prostate (15). TRPM-2 mRNA was not detected in sham castrated controls, but was strongly expressed at 24 h following castration, with mRNA levels increasing up to 72 h (Figure 2A). At 6 h after castration, TRPM-2 mRNA is absent in the ventral prostate although, as shown in Figure 1, extensive apoptosis is already seen at this time. This suggests that TRPM-2 is induced during prostate involution, but does not initiate

apoptosis in the gland following castration.

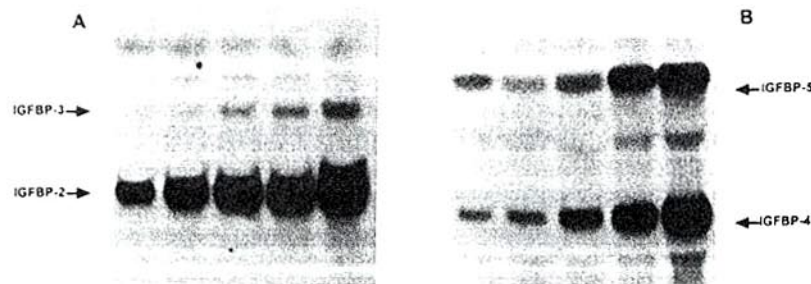
#### *Effects of castration on IGFBP expression in the ventral prostate*

Castration resulted in increased expression of IGFBP-2,-3,-4 and -5 in the ventral prostate. IGFBP-2 mRNA is abundant in the normal prostate of sham castrated controls, increases 3-fold by 6 h and reaches 9-fold by 72 h in the prostate of castrated rats (Figure 3A). IGFBP-3 mRNA is expressed at a very low level in control animals and is increased 1.3-fold and 10-fold 6 h and 72 h respectively after castration (Figure 3A). IGFBP-4 and -5 mRNAs are both expressed in the normal prostate and increase 1.5-fold and 1.3-fold respectively by 6 h, and 8-fold and 5-fold



**Figure 2.** Effects of castration on TRPM-2/clusterin gene expression in the ventral prostate. The 2.3 kb TRPM-2/clusterin transcript detected by Northern analysis is indicated (A). One representative filter was used for all Northern blots shown, and ethidium bromide staining of the agarose gel is shown (B).





**Figure 3.** Expression of IGFBP's in the rat ventral prostate following castration. The 2.6 kb and 1.4 kb mRNAs for IGFBP-3 and -2 respectively were detected by Northern blot analysis as described in Materials and Methods and are indicated (A). The filter was stripped and re-probed to detect the 6.0 kb IGFBP-5 mRNA and 2.4 kb IGFBP-4 mRNA (B). Expression of IGFBP genes was quantitated by densitometric scanning of the autoradiograms.

respectively by 72 h following castration (Figure 3B). All differences were statistically significant ( $p < 0.05$ ) as determined by Mann-Whitney U-test.

#### Effects of castration on IGF-I gene expression and on binding of $^{125}$ I-IGF-I to the IGF-I receptor

IGF-I gene expression increases 6 h after castration (Figure 4A). However, this is followed by a rapid decrease in IGF-I mRNA levels in the ventral prostate of castrated animals which by 72 h is 35% of that seen in sham castrated controls. Similarly, IGF-IR binding capacity, as determined by affinity labelling experiments, increases initially after castration, but then falls to below control levels (Figure 4B). Expression of IGF-II was not detected in either control or castrated animals (data not shown).

#### Discussion

It had previously been hypothesized that IGFBP-5 serves to trigger apoptosis in the prostate following androgen ablation (20). Our data support this hypothesis by demonstrating that induction of IGFBP's in the rat ventral prostate after castration is rapidly and closely associated with induction of apoptosis in the gland. These observations provide the first demonstration that expression of IGFBP-2, -3, -4 and -5 increase rapidly in the ventral prostate following castration, and suggest that IGFBP's may play a role in apoptosis during involution of the prostate. This may involve reduction of IGF binding to IGF receptors and/or direct apoptotic inducing mechanisms. Although we observe increased expression of IGFBP's -2, -3, -4 and -5 in the prostate after castration, IGFBP-2 is unlikely to be important for apoptosis of the prostate gland based on its constitutive high expression in intact animals, but may facilitate the actions of other IGFBP's (20). IGFBP-5 expression has previously been shown to be dramatically increased during post-lactational involution of the rat mammary gland (21). Our observation that IGFBP-5 mRNA increases in the ventral prostate 2-fold by 24 h and 5-fold by 72 h after castration is in keeping with the hypothesis that IGFBP-5 may be involved in apoptosis resulting from steroid hormone deprivation.

IGFBP-3 is also likely to be important for apoptosis of the prostate during involution. The expression of IGFBP-3 mRNA in the normal prostate of sham castrated controls is very low and IGFBP-3 mRNA levels increase 1.3-fold by 6 h and 10-fold by 72 h post-castration. Furthermore, it has recently been demonstrated that IGFBP-3 induces apoptosis in PC-3 prostate carcinoma cells (12) as well as in MCF7 breast cancer cells (14).

IGFBP-3 has been reported to mediate the growth inhibitory effects of TGF- $\beta$  in various cell types (22,23) and to mediate

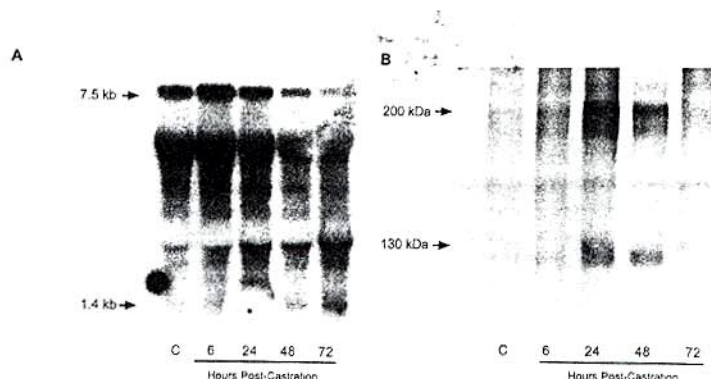
apoptosis induced by TGF- $\beta$  in PC-3 cells (12). We found that castration raised IGFBP-3 mRNA abundance prior to appearance of TGF- $\beta$  mRNA, which is only detected at 24 h (data not shown). This is consistent with a previous report that TGF- $\beta$  is under negative androgenic regulation in the rat ventral prostate and is expressed 24 h after castration (24), but supports the hypothesis that IGFBP-3 plays a role in castration-induced apoptosis in the rat prostate.

Rapid induction of IGFBP's following castration may sequester IGF-I away from the IGF-I receptor (20). This might be expected to result in compensatory upregulation of IGF-I expression and/or IGF-I receptor levels. Indeed, we observed an initial increase in IGF-I gene expression together with a slightly delayed increase in IGF-IR binding, followed by declines in both.

Our previous findings on IGFBP-3 induced apoptosis *in vitro* (14) were consistent with the hypothesis that IGFBP's regulate apoptosis by modulating IGF bioavailability. There is growing evidence that IGFBP-3 has effects on cell growth and survival that occur via IGF-independent mechanisms which are mediated by a putative IGFBP-3 receptor ((25,26) reviewed in (11)). Ligand blotting of the membrane fraction isolated from rat prostate tissue with  $^{125}$ I-IGFBP-3 revealed discrete bands at 18, 45 and 87 kDa which bind IGFBP-3 (data not shown), although the molecular weights are not identical to bands reported by Rajah et al (12) from PC-3 cells which bind IGFBP-3 with high affinity. In view of this evidence that the rat ventral prostate exhibits potential binding sites for IGFBP-3 on cell membranes, we can not exclude the possibility that IGFBP-3 exerts direct regulatory effects in this tissue.

We have shown that increased expression of IGFBP mRNA is temporally related to induction of apoptosis in the ventral prostate following castration. However, our experimental system does not allow us to prove whether IGFBP's cause apoptosis in the ventral prostate or are upregulated as a result of apoptosis. To further elucidate the role of IGFBP's in apoptosis induced by hormone deprivation it will be necessary to examine interactions at the protein level, but such studies must await availability of suitable antibodies against rat IGF binding proteins. Formal demonstration using IGFBP knockout models that induction of IGFBP's is required for castration-induced prostate gland apoptosis to occur may be challenging, as our data indicate that multiple IGFBP's are involved. It may be necessary to simultaneously disrupt multiple IGFBP genes to determine to what extent effects of castration on the prostate gland are a consequence of induction of expression of IGFBP's.





**Figure 4.** Effects of castration on IGF-I gene expression and IGF-I receptor binding in the rat ventral prostate following castration. IGF-I mRNA transcripts were detected by Northern blot analysis (A). For each lane, the sum of the density bands corresponding to IGF-I transcripts was calculated. Membrane extracts prepared from rat ventral prostate were used for IGF-IR affinity labelling as described in Materials and Methods (B).

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